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Biosynthetic Intermediate Analysis and Functional Homology Reveal a Saxitoxin Gene Cluster in Cyanobacteria▽†

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Saxitoxin (STX) and its analogues cause the paralytic shellfish poisoning (PSP) syndrome, which afflicts human health and impacts coastal shellfish economies worldwide. PSP toxins are unique alkaloids, being produced by both prokaryotes and eukaryotes. Here we describe a candidate PSP toxin biosynthesis gene cluster (*sxt*) from *Cylindrospermopsis raciborskii* T3. The saxitoxin biosynthetic pathway is encoded by more than 35 kb, and comparative sequence analysis assigns 30 catalytic functions to 26 proteins. STX biosynthesis is initiated with arginine, *S*-adenosylmethionine, and acetate by a new type of polyketide synthase, which can putatively perform a methylation of acetate, and a Claisen condensation reaction between propionate and arginine. Further steps involve enzymes catalyzing three heterocyclizations and various tailoring reactions that result in the numerous isoforms of saxitoxin. In the absence of a gene transfer system in these microorganisms, we have revised the description of the known STX biosynthetic pathway, with in silico functional inferences based on *sxt* open reading frames combined with liquid chromatography-tandem mass spectrometry analysis of the biosynthetic intermediates. Our results indicate the evolutionary origin for the production of PSP toxins in an ancestral cyanobacterium with genetic contributions from diverse phylogenetic lineages of bacteria and provide a quantum addition to the catalytic collective available for future combinatorial biosyntheses. The distribution of these genes also supports the idea of the involvement of this gene cluster in STX production in various cyanobacteria.

Paralytic shellfish poisoning (PSP) toxins are among the world's most potent and pervasive toxins and are considered a serious toxicological health risk that may affect humans, animals, and ecosystems worldwide (18, 36). These toxins block voltage-gated sodium and calcium channels and prolong the gating of potassium channels (21, 53, 59), preventing the transduction of neuronal signals. It has been estimated that more than 2,000 human cases of PSP occur globally every year at a mortality rate of 15% (16). Moreover, coastal blooms of productive microorganisms result in millions of dollars of economic damage due to PSP toxin contamination of seafood and the continuous requirement for costly biotoxin monitoring programs. Early warning systems to anticipate the occurrence of paralytic shellfish toxin (PST)-producing algal blooms, such as PCR and enzyme-linked immunosorbent assay-based screening, are as yet unavailable due to the lack of data on the genetic basis of PST production.

Saxitoxin (STX) is a tricyclic perhydropurine alkaloid that can be substituted at various positions, leading to more than 30 naturally occurring STX analogues (4, 5, 28, 32, 33, 63). Although STX biosynthesis seems complex and unique, organisms from two kingdoms, including certain species of marine dinoflagellates and freshwater cyanobacteria, are capable of producing these toxins, apparently by the same biosynthetic route (47). In spite of considerable efforts, none of the enzymes or genes involved in the biosynthesis and modification of STX have been previously identified (15, 39, 40, 44, 62). Here, based on previously published knowledge regarding the possible steps in STX biosynthesis (47), together with information from our recent in vitro biosynthesis of STX (22), we used an approach that employed reverse genetics to identify the candidate STX biosynthetic gene cluster (*sxt*) in the cyanobacterium *Cylindrospermopsis raciborskii* T3 (23). Since this organism is not genetically transformable, mutagenic characterization of the *sxt* cluster was not possible. However, here we present the bioinformatically inferred functions for most of the open reading frames (ORFs) in this gene cluster and provide evidence for their role in STX metabolism via liquid chromatography-tandem mass spectrometry (LC–MS-MS) screening of the biosynthetic intermediates in concentrated cell extracts of *C. raciborskii* T3. The in silico functional assignment of *sxt* genes and the chemical detection of STX biosynthesis intermediates have enabled a thorough revision of previous knowledge concerning the known STX biosynthetic pathway.

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MATERIALS AND METHODS

Cyanobacterial cultures. Cyanobacterial strains used in the present study (Table 1) were grown in Jaworski medium (55) in static batch culture at 26°C under conditions of continuous illumination (10 μmol m^{-2} s^{-1}) with fluorescent cool-white light.

Characterization of the *sxt* gene cluster. Total genomic DNA was extracted from cyanobacterial cells by lysozyme/sodium dodecyl sulfate/proteinase K lysis

TABLE 1. Distribution of the *sxt* genes in toxic and nontoxic cyanobacteria[a]

Cyanobacterium strain	Toxicity (reference)	Presence or absence of gene (PCR product accession number)[b]				
		sxtA	*sxtG*	*sxtH*	*sxtI*	*sxtX*
A. circinalis AWQC118C	PSP (56)	+ (EU629177)	+ (EU629183)	+ (EU629197)	+ (EU439560)	—
A. circinalis AWQC131C	PSP (25)	+ (EU629179)	+ (EU629190)	+ (EU629194)	+ (EU439557)	—
A. circinalis AWQC134C	PSP (56)	+	+ (EU629186)	+ (EU629195)	+ (EU439561)	—
A. circinalis AWQC150E	PSP (56)	+ (EU629176)	+ (EU629187)	+ (EU629199)	+ (EU439563)	—
A. circinalis AWQC173A	PSP (56)	+	+ (EU629188)	+ (EU629198)	+ (EU439564)	—
A. circinalis AWQC271C	— (56)	—	—	—	—	—
A. circinalis AWQC306A	— (56)	—	—	—	—	—
A. circinalis AWQC310F	— (56)	—	—	—	—	—
A. circinalis AWQC342D	— (56)	—	—	—	—	—
Aphanizomenon flos-aquaea NH-5	PSP (26)	+ (EU629175)	+ (EU629181)	+ (EU629192)	+ (EU439559)	+ (EU629201)
Aphanizomenon ovalisporum APH028A	CYLN[c] (46)	—	—	—	—	—
C. raciborskii T3	PSP (23)	+ (EU629178)	+ (EU629182)	+ (EU629193)	+ (EU439556)	+ (EU629202)
C. raciborskii 23B	CYLN (60)	—	—	—	—	—
C. raciborskii GOON	CYLN (43)	—	—	—	—	—
C. raciborskii GERM1	— (30)	—	—	—	—	—
C. raciborskii MARAU1	— (30)	—	—	—	—	—
L. wollei	PSP (7)	+ (EU629174)	+ (EU629180)	+ (EU629191)	+ (EU439558)	+ (EU629200)

[a] The presence of genes was determined by PCR and the identity of PCR products confirmed by sequencing.
[b] +, gene fragment amplified; —, no gene detected. NCBI accession numbers of sequenced PCR products are given.
[c] CYLN, cylindrospermopsin.

following phenol-chloroform extraction as described previously (29). DNA in the supernatant was precipitated with 2 volumes of −20°C ethanol, washed with 70% ethanol, dissolved in Tris-EDTA buffer (10:1), and stored at −20°C. In a previous study (R. Kellmann, T. K. Mihali, and A. Neilan Brett, submitted for publication), a gene (*sxt1*) was identified that was exclusively present in PSP-toxic strains of cyanobacteria. It encoded an *O*-carbamoyltransferase that was predicted to catalyze the formation of the *O*-carbamoyl side chain of saxitoxin. Adaptor-mediated PCR (49), with modifications as described by Moffitt and Neilan (27), was used to determine the sequence of genes adjacent to *sxt1*. Short adaptor DNA was ligated to restriction enzyme-digested genomic DNA, and a specific genomic outward-facing primer was then used with an adaptor primer to amplify a region of the genome. Twenty picomoles of T7 adaptor was added to each reaction mixture, containing 1 μg of genomic DNA, 10 U of blunt-ended restriction enzyme, and 5 U of T4 ligase (Promega) in 1× One-Phor-All buffer (Amersham/Pharmacia). The one-step digestion and ligation reaction mixture was incubated at room temperature overnight. The single-stranded end of the adaptor was blocked in a solution containing 1× PCR buffer (Fischer Biotech), 4 mM $MgCl_2$, and 12.5 μM dideoxynucleotide triphosphate with 1 U of *Taq* DNA polymerase (Fischer Biotech). Thermal cycling was performed using a PCR Sprint temperature cycling system machine (Hybaid Limited) with an initial step at 70°C for 15 min followed by 10 cycles of DNA denaturation at 95°C for 10 s, DNA reannealing at 40°C for 1 min, and extension of the strand with dideoxynucleotide triphosphate at 70°C for 1 min. Following the PCR cycles, the reaction mixture was incubated with 1 U of shrimp alkaline phosphatase (Boehringer Mannheim, Göttingen, Germany) at 37°C for 20 min, and the enzyme was heat inactivated at 85°C for 5 min.

The flanking-region PCR mixture contained 1 to 2 μl of adaptor-ligated DNA, 10 pmol of adaptor primer, and 10 pmol of a genome-specific oligonucleotide primer. Primer sequences are given in the supplemental material. PCR cycling was performed as described above, with DNA strand extension at 72°C for 5 min. The primer annealing temperature was decreased (from 65 to 55°C) by 1°C at each cycle, followed by primer annealing at 55°C for a further 25 cycles. PCR amplicons were separated by agarose gel electrophoresis using TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 7.8) and visualized by UV translumination after staining in ethidium bromide (0.5 μg/ml). PCR amplicons were excised and purified for sequencing as described below.

Automated DNA sequencing was performed using a PRISM BigDye cycle sequencing system and a model 373 sequencer (Applied Biosystems). Sequence data were analyzed using ABI Prism Autoassembler software, and percent similarity and identity to other translated sequences were determined using BLAST in conjunction with the National Center for Biotechnology Information (NIH). The *sxt* gene clusters were assembled using the software Phred, Phrap, and Consed (http://www.phrap.org/phredphrapconsed.html), and ORFs were manually identified. The correctness of the assembly was verified by PCR using genomic DNA as the template, genome-specific primer pairs that targeted overlapping sequence stretches along the entire *sxt* gene cluster, and sequencing of the obtained PCR amplicons.

MS analysis of STX intermediates. Extracts were prepared from the cell mass from 1-liter cultures of the PSP-toxic *C. raciborskii* strain T3 and the non-PSP-toxic *C. raciborskii* strain AWT205. Cells were lysed in a French press, and the lysate was centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant was filtered using a spin filter with a nominal molecular mass cutoff of 10,000 Da. Bacterial extracts and STX standards were analyzed by high-performance liquid chromatography (HPLC; Thermo Finnigan Surveyor and autosampler) coupled to an ion trap mass spectrometer (Thermo Finnigan LCQ Deca XP Plus) fitted with an electrospray source. Separation of analytes was obtained on a Phenomenex Luna 3-μm C_{18} column (2.1 mm by 150 mm) at 100 ml/min. Analysis was performed using a gradient starting at 95% water –10 mM heptafluorobutyric acid in water (buffer A). This mixture was maintained for 10 min and was then ramped to a 100% organic mixture (acetonitrile [buffer B]) over 30 min. Conditions were maintained using 100% buffer B for 10 min to wash the column, and then the conditions were returned to use of 100% buffer A and again maintained for 10 min to equilibrate the column for the next sample. This resulted in a runtime of 60 min per sample. Sample volumes of 10 to 100 ml were injected for each analysis. The HPLC eluate directly entered the electrospray source, which was programmed as follows: electrospray voltage, 5 kV; sheath gas flow rate, 30 arbitrary units; and auxiliary gas flow rate, 5 arbitrary units. The capillary temperature was 200°C and had a voltage of 47 V. Ion optics were optimized for maximum sensitivity before sample analysis was performed using the autotune function of the instrument with a standard toxin solution. Mass spectra were acquired in the centroid mode over an *m/z* range of 145 to 650. The mass range was set at "normal," with 200 ms of maximum ion injection time and automatic gain control engaged. Tandem mass spectra were obtained over an *m/z* range relevant to the precursor ion. Collision energy was typically 20 to 30 ThermoFinnigan arbitrary units and was optimized for maximal information, using standards where available.

Nucleotide sequence accession number. Nucleotide sequences were submitted to GenBank and are available under accession number DQ787200.

RESULTS AND DISCUSSION

Identification and sequencing of the *sxt* gene cluster in *C. raciborskii* T3. A biosynthetic pathway has been previously proposed based on the pattern of substrate incorporation into

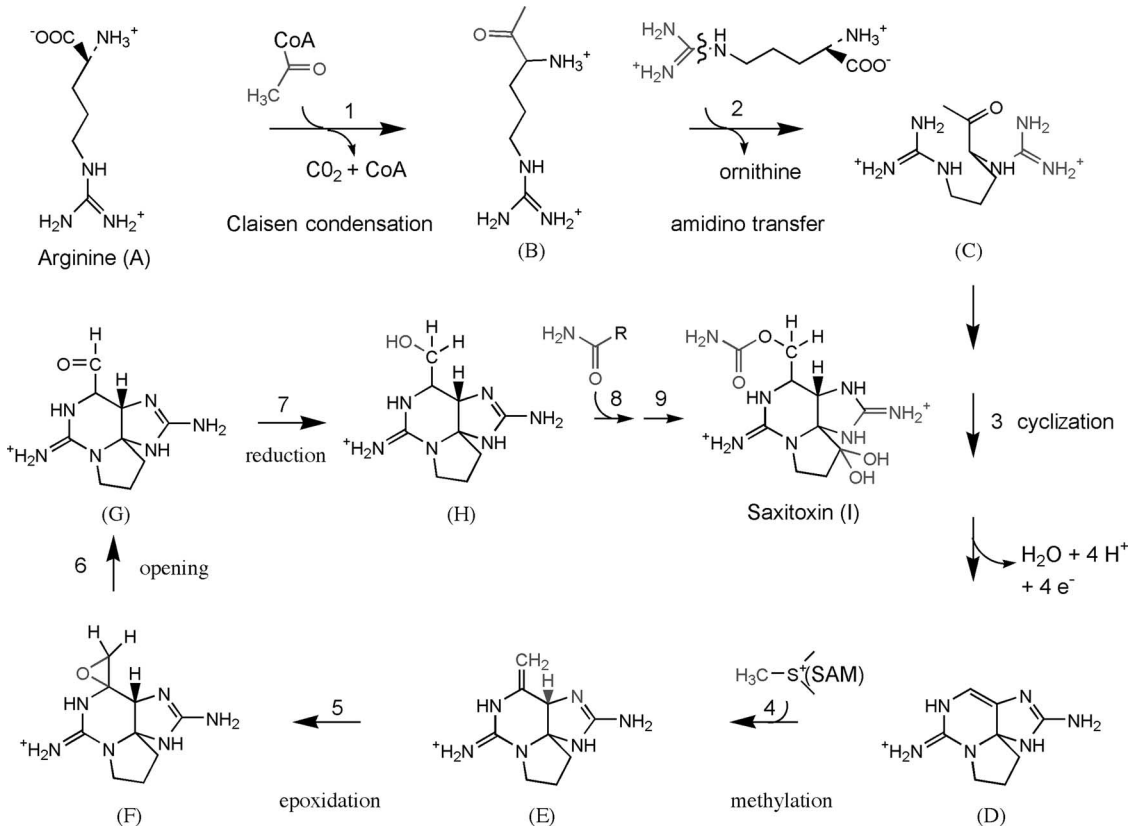

FIG. 1. Hypothetical biosynthesis pathway of STX as proposed by Shimizu et al. (47). Hypothetical intermediate metabolites are labeled with letters in brackets. The reaction steps are as follows: 1, Claisen condensation reaction between acetate and arginine; 2, amidino transfer from a second arginine to the α-amino group of intermediate B; 3, cyclization; 4, introduction of SAM methyl-derived side chain, involving the loss of one methionine methyl hydride; 5, epoxidation of side chain, leading to a 1,2-H shift; 6, opening of epoxide to an aldehyde followed by reduction of the aldehyde; 7 and 8, carbamoyl transfer and dihydroxylation.

STX, suggesting arginine, acetate (via acetyl-coenzyme A [acetyl-CoA]), and methionine methyl (via *S*-adenosylmethionine [SAM]) as precursors (47). The first and most unusual of these reactions has been suggested to be a Claisen condensation of arginine and acetate (48). Claisen condensations on amino acids are rare, but do occur, for instance, in the biosynthesis of tetrapyrroles, biotin, and arphamenine AB and in the conversion between glycine, serine, and threonine (2). This reaction is then followed by the transfer of a guanidino group from a second arginine to the first intermediate. The three heterocycles of STX are formed by unknown reactions, and the resulting intermediate is methylated to form a side chain, which is hydroxylated via an epoxide and then *O*-carbamoylated. Finally, one of the ring carbons (C-12) receives two hydroxy groups (Fig. 1).

This previously proposed pathway for the biosynthesis of STX suggested the involvement of enzymes with the catalytic activities of a class II aminotransferase, amidinotransferase, SAM-dependent methyltransferase, hydroxylases, and *O*-carbamoyltransferase. Our recent in vitro biosynthesis studies (22) confirmed arginine, acetyl-CoA, SAM, and carbamoylphosphate as precursors for STX biosynthesis, further supporting the involvement of an *O*-carbamoyltransferase in the biosynthesis of STX. When we screened for putative STX-biosynthetic enzymes in cyanobacterial isolates, a gene encoding an *O*-carbamoyltransferase in *C. raciborskii* T3 was initially detected via degenerate PCR (Kellmann et al., submitted). Further investigation showed that homologues of *sxtI* were exclusively present in PSP toxin-producing strains of four cyanobacterial genera (Table 1); thus, *sxtI* represents a good candidate gene for PSP toxin biosynthesis. The sequence of the complete putative STX biosynthetic gene cluster (*sxt*) was then obtained by genome walking up- and downstream of *sxtI* in *C. raciborskii* T3 (Fig. 2). In *C. raciborskii* T3, this *sxt* gene cluster spans approximately 35,000 bp, encoding 31 ORFs (Table 2). The cluster also included other genes encoding previously predicted STX-biosynthesis enzymes, including a methyltransferase (*sxtA1*), a class II aminotransferase (*sxtA4*), an amidinotransferase (*sxtG*), and dioxygenases (*sxtH*), in addition to the *O*-carbamoyltransferase (*sxtI*). PCR screening of selected *sxt* ORFs in a total of 18 cyanobacterium strains showed that they were exclusively present in PSP toxin-producing isolates (Table 1), indicating the association of these genes with the toxic phenotype. In the following passages we describe the ORFs in the putative *sxt* gene cluster and their predicted functions, based on bioinformatic analysis and LC–MS-MS data on biosynthetic intermediates and in vitro biosynthesis, when applicable.

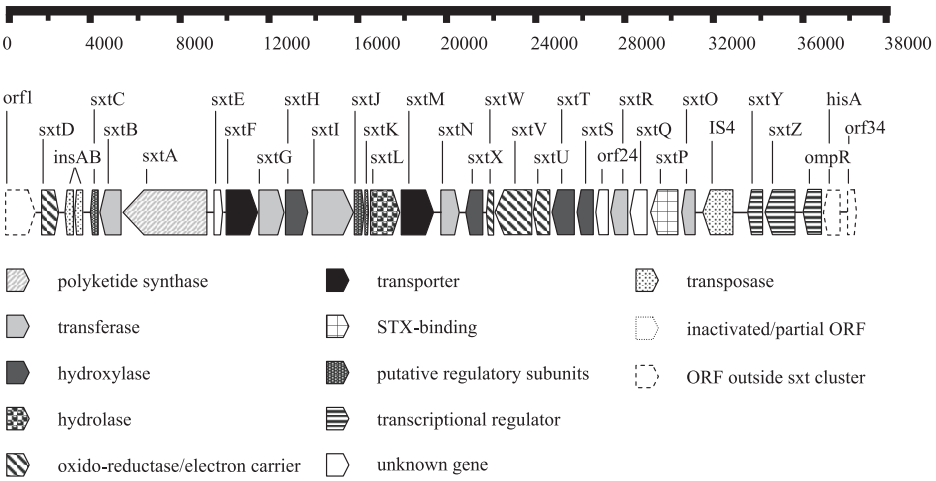

FIG. 2. Structural organization of the *sxt* gene cluster from *C. raciborskii* T3. Abbreviations used are as follows: IS4, insertion sequence 4; ompR, transcriptional regulator of *ompR* family; hisA, two-component histidine kinase; orf24, ORF 4. The scale indicates the gene cluster lengths in base pairs.

Functional identification of the parent molecule biosynthetic genes. Bioinformatic analysis of the *sxt* gene cluster revealed that it contains a previously undescribed example of a polyketide synthase (PKS)-like structure named *sxtA*. SxtA possesses four catalytic domains, SxtA1 to SxtA4. An iterated PSI-BLAST search revealed low sequence homology of SxtA1 to SAM-dependent methyltransferases. Further analysis revealed the presence of three conserved sequence motifs in SxtA1 (278-ITDMGCGDG-286, 359-DPENILHI-366, and 424-VVNKHGLMIL-433) that are specific for SAM-dependent methyltransferases (19). SxtA2 is related to GCN5-related *N*-acetyl transferases (GNAT). GNAT catalyze the transfer of acetate from acetyl-CoA to various heteroatoms (31) and have been reported in association with other unconventional PKSs, such as PedI (37), where they load the acyl carrier protein (ACP) with acetate. SxtA3 is related to an ACP and provides a phosphopantetheinyl-attachment site (24). SxtA4 is homologous to class II aminotransferases and was most similar to AONS (8-amino-7-oxononanoate synthase). Class II aminotransferases are a monophyletic group of pyridoxal phosphate-dependent enzymes and are the only enzymes that are known to perform Claisen condensation of amino acids (2). In vitro biosynthesis of STX in *C. raciborskii* has also been shown to be dependent on the presence of pyridoxal phosphate (22). We therefore reasoned that *sxtA* performs the first step in STX biosynthesis, involving a Claisen condensation reaction.

It has been proposed that acetate is condensed to arginine in the first step of STX biosynthesis, while the methyl side chain is introduced during a latter step (47). However, the predicted reaction sequence of SxtA, based on its primary structure, is the loading of the ACP (SxtA3) with acetate from acetyl-CoA, followed by the SxtA1-catalyzed methylation of acetyl-ACP, converting it to propionyl-ACP. SxtA4, the class II aminotransferase domain, would then perform a Claisen condensation reaction between propionyl-ACP and arginine (Fig. 3). The putative product of SxtA is thus 4-amino-3-oxo-guanidinoheptane, which is here designated compound A′ (Fig. 3). This sequence of reactions in the first step of STX biosynthesis is inconsistent with that proposed by Shimizu (47) (Fig. 1). To verify this revised pathway for STX biosynthesis based on comparative gene sequence analysis, cell extracts of *C. raciborskii* T3 were screened by LC–MS-MS for the presence of compound A′ (Fig. 4) and compound B, as predicted by Shimizu (47) (Fig. 1), as well as for arginine and STX as controls. In addition, LC–MS-MS analysis was performed on cell extracts from the non-PSP-toxic strain *C. raciborskii* AWT205. While arginine and STX were readily detected in extracts from *C. raciborskii* T3 (Fig. 4) and produced the expected fragment ions (50, 58), there were no measurable levels of compound B. On the other hand, LC–MS-MS data obtained at an *m*/*z* of 187 were consistent with the presence of structure A′ from *C. raciborskii* T3 (Fig. 4). MS-MS spectra showed the expected fragment ion (*m*/*z* 170 and 128) (58) after the loss of ammonia and guanidine from structure A′. Arginine was readily detected in cell extracts from *C. raciborskii* AWT205; however, there were no measurable levels of STX, compound A′, or compound B. LC–MS-MS data strongly supported the predicted function of SxtA and, thus, a revised initiating reaction in the STX biosynthesis pathway.

sxtG encodes a putative amidinotransferase, which had the highest amino acid sequence similarity to L-arginine/L-lysine amidinotransferases. We propose that the product of SxtA is the substrate for the amidinotransferase SxtG, which transfers an amidino group from arginine to the α-amino A′ group (Fig. 3), thus producing 4,7-diguanidino-3-oxoheptane (designated compound B′) (Fig. 3). The description of this hypothetical sequence of reactions was also supported by the detection of compound C′ by LC–MS-MS (Fig. 4). Cell extracts from *C. raciborskii* T3, however, did not contain any measurable levels of B′ (4,7-diguanidino-3-oxoheptane). A likely explanation for the failure to detect the intermediate B′ is its rapid cyclization to form C′ via the action of SxtB. Compound B′ and compound C′ were not detected in extracts from *C. raciborskii* AWT205.

The *sxt* gene cluster encodes SxtB, an enzyme similar to the cytidine deaminase-like enzymes from gammaproteobacteria.

TABLE 2. The *sxt* genes from *C. raciborskii* T3 and their predicted functions

Gene	Enzyme family	Size (bp)	BLAST similarity match	% Similarity	Putative function
orf1	Unknown protein	1,320	BAB76734.1 *Nostoc* sp. strain PCC7120	82	Unknown
sxtD	Sterole desaturase-like protein	759	ABG52264.1 *Trichodesmium erythraeum*	63	Desaturation
orf3	Transposase InsB	392	CAE11915.2 *Microcystis aeruginosa*	86	Transposition
orf4	Transposase InsA	360	CAE11914.1 *Microcystis aeruginosa*	71	Transposition
sxtC	Unknown protein	354	No similarity found		Regulatory
sxtB	Cytidine deaminase	957	EAS64681.1 *Vibrio angustum*	62	Cyclization
sxtA	Methyltransferase	1,506	ABF89568.1 *Myxococcus xanthus*	64	Methylation
	GNAT	633	AAT70096.1 CurA *Lyngbya majuscula*	64	Loading of ACP
	Acyl carrier protein	330	AAV97870 OnnB *Theonella swinhoei*	59	ACP
	AONS	1,236	ABD13093.1 *Frankia* sp. strain Ccl3	61	Claisen condensation
sxtE	Unknown protein	387	ABE53436.1 *Shewanella denitrificans*	52	Unknown
sxtF	MATE	1,416	NorM ABC44739.1 *Salinibacter ruber*	52	Export of PSTs
sxtG	Amidinotransferase	1,134	ABA05575.1 *Nitrobacter winogradskyi*	71	Amidinotransfer
sxtH	Phenylpropionate dioxygenase	1,005	ZP_00243439.1 *Rubrivivax gelatinosus*	50	C-12 hydroxylation
sxtI	Carbamoyltransferase	1,839	ABG50968.1 *Trichodesmium erythraeum*	82	Carbamoylation
sxtJ	Unknown protein	444	EAM51043.1 *Crocosphaera watsonii*	72	Regulatory
sxtK	Unknown protein	165	ABG50954.1 *Trichodesmium erythraeum*	81	Regulatory
sxtL	GDSL-lipase	1,299	ABG50952.1 *Trichodesmium erythraeum*	60	Decarbamoylation
sxtM	MATE	1,449	NorM ABC44739.1 *Salinibacter ruber*	53	Export of PSTs
sxtN	Sulfotransferase	831	ABG53102.1 *Trichodesmium erythraeum*	57	Sulfotransfer
sxtX	Cephalosporin hydroxylase	774	ABG50679.1 *Trichodesmium erythraeum*	77	N-1 hydroxylation
sxtW	Ferredoxin	327	ZP_00106179.2 *Nostoc punctiforme*	99	Electron carrier
sxtV	Succinate dehydrogenase	1,653	ABA24604.1 *Anabaena variabilis*	92	Dioxygenase reductase
sxtU	Alcohol dehydrogenase	750	ZP_00111652.1 *Nostoc punctiforme*	83	Reduction of C-1
sxtT	Phenylpropionate dioxygenase	1,005	ZP_00243439.1 *Rubrivivax gelatinosus*	48	C-12 hydroxylation
sxtS	Phytanoyl-CoA dioxygenase	726	ABG30370.1 *Roseobacter denitrificans*	41	Ring formation
orf24	Unknown protein	576	No similarity found		Unknown
sxtR	Acyl transferase	777	AAU26161.1 *Legionella pneumophila*	54	Unknown
sxtQ	Unknown protein	777	EAR64935.1 *Bacillus* sp. strain NRRL B-14911	46	Unknown
sxtP	RTX toxin	1,227	ABA20206.1 *Anabaena variabilis*	68	Binding of PSTs
sxtO	Adenylylsulfate kinase	603	ZP_00053494.2 *Magnetospirillum magnetotacticum*	76	PAPS biosynthesis
orf29	Transposase, IS4	1,350	EAO22567.1 *Syntrophobacter fumaroxidans*	61	Transposition
sxtY	PhoU	666	BAB76200.1 *Nostoc* sp. strain PCC7120	87	Signal transduction
sxtZ	Histidine kinase	1,353	ABA22975.1 *Anabaena variabilis*	78	Signal transduction
ompR	OmpR	819	ZP_00108178.2 *Nostoc punctiforme*	91	Signal transduction
hisA	PROFAR isomerase	774	ABA22979.1 *Anabaena variabilis*	90	Histidine biosynthesis
orf34	Unknown protein	396	ZP_00345366.1 *Nostoc punctiforme*	84	Unknown

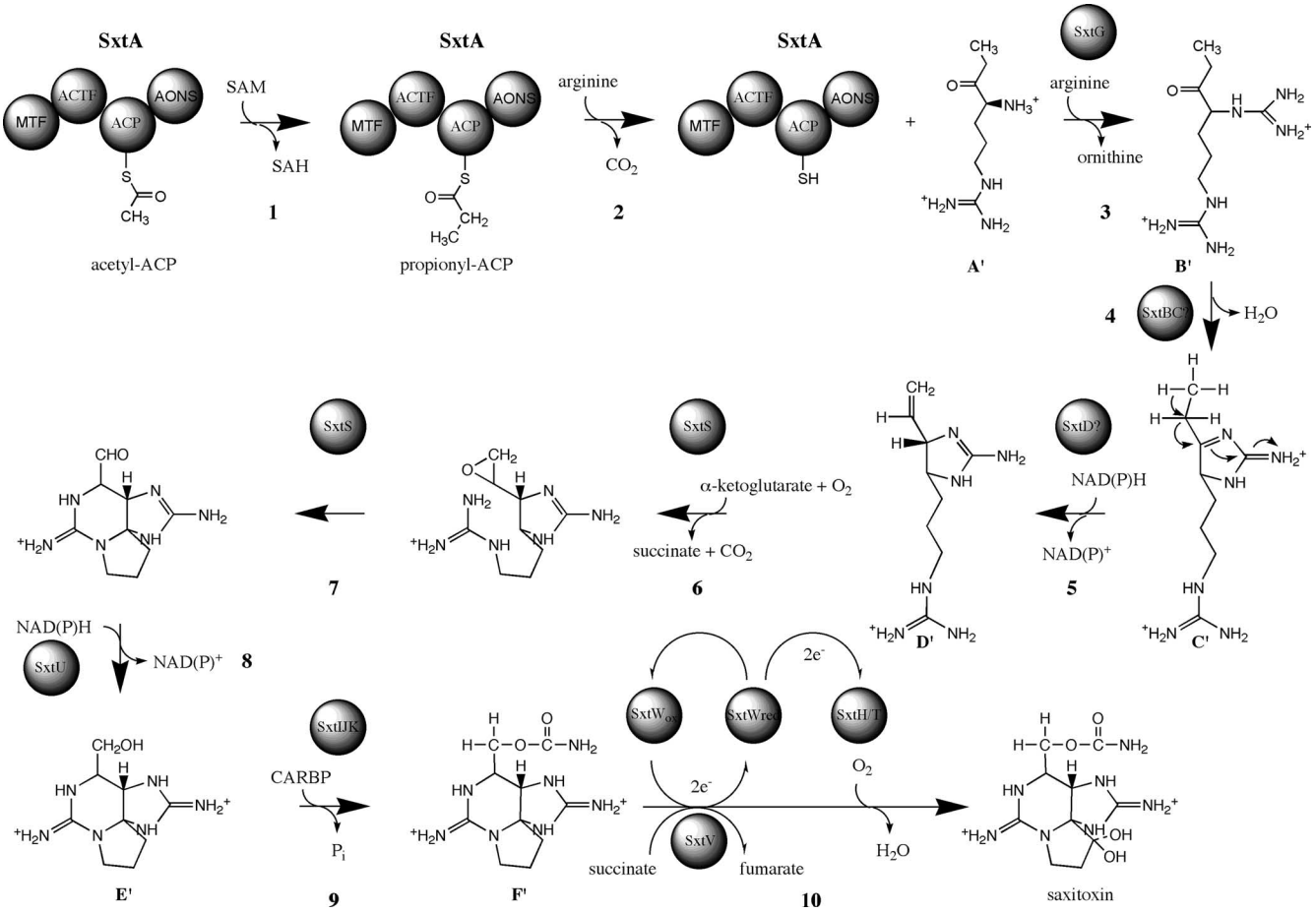

FIG. 3. Revised pathway for STX biosynthesis and the putative functions of *sxt* genes (see text for description).

The catalytic mechanism of cytidine deaminase is a retroaldol cleavage of ammonia from cytidine, which is the same reaction mechanism in the reverse direction as that seen in the formation of the first heterocycle in the conversion from B′ to C′ (Fig. 3). We therefore suggest that SxtB catalyzes this retroaldol-like condensation (see step 4 in Fig. 3).

The incorporation of methionine methyl into STX and its hydroxylation have been studied in detail. Only one methionine methyl-derived hydrogen is retained in STX, and a 1,2-H shift has been observed between acetate-derived C-5 and C-6 of STX. The results of labeled precursor feeding studies indicated that hydroxylation of the methyl side chain of the STX precursor proceeds via epoxidation of a double bond between the SAM-derived methyl group and the acetate-derived C-6 (see steps 5 to 7 in Fig. 1). Shimizu (47) proposed that this incorporation pattern resulted from an electrophilic attack of methionine methyl (see step 4 in Fig. 1) on the double bond between C-5 and C-6, which would have formed during the preceding cyclization (see step 3 in Fig. 1). Subsequently, the new methylene side chain would be epoxidated, followed by opening to an aldehyde and subsequent reduction to a hydroxyl (see steps 5 to 7 in Fig. 1). The point of contention concerning this scheme is that it would also result in a 1,2-H shift between C-1 and C-5, which has not been observed (47). Nevertheless, the retention of only one methionine methyl-derived hydrogen, the 1,2-H shift between C-5 and C-6, and the lack of a 1,2-H shift between C-1 and C-5 are entirely consistent with our genetic revision of the STX-biosynthesis pathway, whereby the introduction of methionine methyl precedes the formation of the three heterocycles.

sxtD encodes an enzyme with sequence similarity to sterol desaturase and is the only candidate desaturase present in the *sxt* gene cluster. SxtD is predicted to introduce a double bond between C-1 and C-5 of C′, resulting in the 1,2-H shift between C-5 and C-6 (compound D′; Fig. 3). The gene product of *sxtS* has sequence homology to nonheme iron 2-oxoglutarate-dependent dioxygenases. These are multifunctional enzymes that can perform hydroxylation, epoxidation, desaturation, cyclization, and expansion reactions (42). 2-Oxoglutarate dioxygenases have been reported to catalyze the oxidative formation of heterocycles (61). SxtS could therefore perform the consecutive epoxidation of the new double bond and opening of the epoxide to an aldehyde with concomitant bicyclization. This would explain the retention of only one methionine methyl-derived hydrogen and the lack of a 1,2-H shift between C-1 and C-5 of STX (see steps 5 to 7 in Fig. 3). SxtU has sequence similarity to short-chain alcohol dehydrogenases. The most similar enzyme with a known function is clavaldehyde dehydrogenase (NCBI accession no. AAF86624), which reduces the terminal aldehyde of clavulanate-9-aldehyde to an alcohol

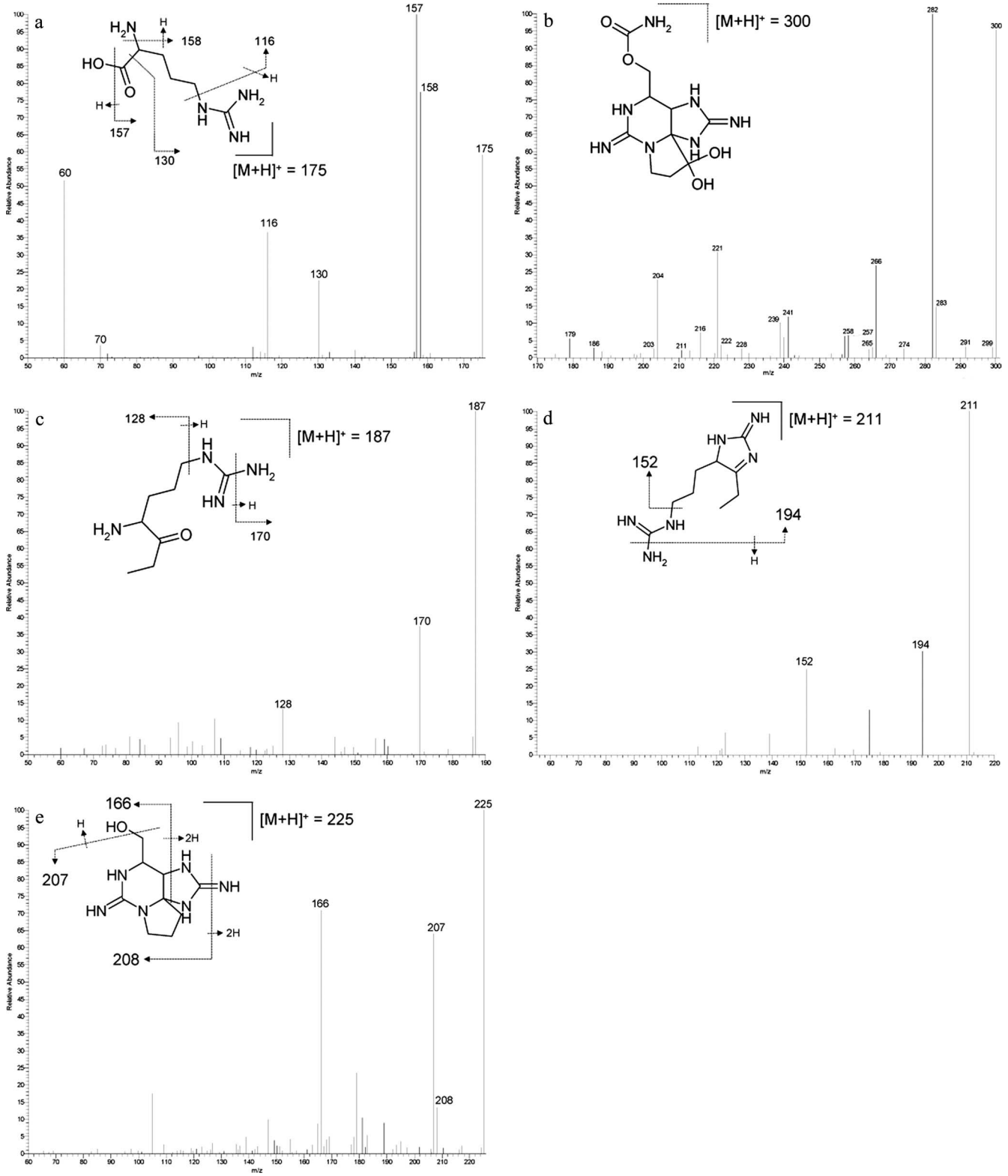

FIG. 4. MS-MS spectra of selected ions from cellular extracts of *C. raciborskii* T3. The predicted fragmentation of ions and the corresponding *m/z* values are indicated. a, arginine (*m/z* 175); b, saxitoxin (*m/z* 300) (refer to Sleno et al. [51] for a more detailed identification and discussion of the fragment ions obtained from the CID of saxitoxin); c, intermediate A′ (*m/z* 187); d, intermediate C′ (*m/z* 211); e, intermediate E′ (*m/z* 225).

(13). SxtU is therefore predicted to reduce the terminal aldehyde group of the STX precursor in step 8 (Fig. 3), forming compound E′.

The concerted action of SxtD, SxtS, and SxtU is therefore responsible for the hydroxylation and bicyclization of compound C′ to E′ (Fig. 3). In support for this proposed revision of STX biosynthesis descriptions, LC–MS-MS obtained using *m*/*z* values of 211 and 225 allowed the detection of compounds C′ and E′ from *C. raciborskii* T3 (Fig. 4). On the other hand, no evidence could be found by LC–MS-MS for intermediates B (*m*/*z*, 216), and C (*m*/*z*, 198), as previously predicted (47) (Fig. 1). MS-MS spectra showed the expected fragment ions (58) after the loss of ammonia and guanidine from C′, as well as the loss of water in the case of E′. Compounds E′ and C were not detected in extracts from *C. raciborskii* AWT205.

The detection of E′ indicated that the final reactions leading to the complete STX molecule are the *O*-carbamoylation of its free hydroxy group and a double hydroxylation of C-12 (Fig. 1). The actual sequence of these final reactions, however, remains uncertain. The gene product of *sxtI* is most similar to a predicted *O*-carbamoyltransferase from *Trichodesmium erythraeum* (accession no. ABG50968) and other predicted *O*-carbamoyltransferases from cyanobacteria. *O*-carbamoyltransferases invariably transfer a carbamoyl group from carbamoylphosphate to a free hydroxyl group. Our data indicate that SxtI may catalyze the transfer of a carbamoyl group from carbamoylphosphate to the free hydroxy group of E′ (Fig. 1). Homologues of *sxtJ* and *sxtK* with a known function were not found in the databases; however, it was noted that *sxtJ* and *sxtK* homologues were often encoded adjacent to *O*-carbamoyltransferase genes.

The *sxt* gene cluster contains two genes, *sxtH* and *sxtT*, each encoding a terminal oxygenase subunit of bacterial phenylpropionate and related ring-hydroxylating dioxygenases. The closest homologue with a predicted function was capreomycidine hydroxylase from *Streptomyces vinaceus* (VioQ; accession no. AAP92508) (54), which hydroxylates a ring carbon (C-6) of capreomycidine. SxtH and SxtT may therefore perform a similar function in STX biosynthesis, that is, the consecutive hydroxylation of C-12, converting F′ into STX (see step 10 in Fig. 1).

Members belonging to bacterial phenylpropionate and related ring-hydroxylating dioxygenases are multicomponent enzymes, as they require an oxygenase reductase for their regeneration after each catalytic cycle. The *sxt* gene cluster provides a putative electron transport system, which would fulfill this function. *sxtV* encodes a 4Fe-4S ferredoxin with high sequence homology to a ferredoxin from *Nostoc punctiforme* (accession no. ZP_00106179). *sxtW* was most similar to fumarate reductase/succinate dehydrogenase-like enzymes from *Anabaena variabilis* (ABA24604) and *Nostoc punctiforme* (accession no. ZP_00106180), followed by AsfA from *Pseudomonas putida* (AAD31780). AsfA and AsfB are enzymes involved in the transport of electrons resulting from the catabolism of aryl sulfonates (57). SxtV could putatively extract an electron pair from succinate, converting it to fumarate (17), and then transfer the electrons via ferredoxin (SxtW) to SxtH and SxtT.

Comparative sequence analysis and functional assignment of tailoring genes. Following synthesis of the parent molecule STX, modifying enzymes introduce various functional groups. In addition to STX, *C. raciborskii* T3 produces N-1-hydroxylated (neoSTX), decarbamoylated (dcSTX), and *N*-sulfurylated (GTX-5) toxins, whereas *Anabaena circinalis* AWQC131C produces decarbamoylated (dcSTX) toxins and *O*-sulfurylated (GTX-3/GTX-2, dcGTX-3/dcGTX-2) toxins, as well as both *O*- and *N*-sulfurylated toxins (C-1/C-2), but no N-1-hydroxylated toxins (25).

sxtX encodes an enzyme with homology to cephalosporin hydroxylase. *sxtX* was detected only in *C. raciborskii* T3, *Aphanizomenon flos-aquae* NH-5, and *Lyngbya wollei*, which produce N-1-hydroxylated analogues of STX (7, 21, 26), such as neoSTX. This component of the gene cluster was not present in any strain of *A. circinalis*, and therefore probably represents the reason why this species does not produce N-1-hydroxylated PSP toxins (25, 56). The predicted function of SxtX is therefore the N-1 hydroxylation of STX.

A. circinalis AWQC131C and *C. raciborskii* T3 also produce *N*- and *O*-sulfated analogues of STX [GTX-5, C-2/C-3, (dc)GTX-3/GTX-4]. The activity of two 3′-phosphate 5′-phosphosulfate (PAPS)-dependent sulfotransferases, which were specific for the N-21 of STX and GTX-3/GTX-2 and the O-22 of 11-hydroxy STX, respectively, has been described previously in studies of the PSP toxin-producing dinoflagellate *Gymnodinium catenatum* (44, 62). The *sxt* gene cluster from *C. raciborskii* T3 encodes SxtN, a putative sulfotransferase. A PSI-BLAST search using SxtN identified only 25 hypothetical proteins of unknown function with an E value above the threshold (0.005). A profile library search, however, revealed significant structural relatedness of SxtN to estrogen sulfotransferase (1AQU) (Z score = 24.02) and other sulfotransferases. SxtN has a conserved N-terminal region, which corresponds to the adenosine PAPS-binding region in 1AQU (20). It is not known, however, whether SxtN transfers a sulfate group to N-21 or O-22. Interestingly, the *sxt* gene cluster encodes an adenylylsulfate kinase (APSK), SxtO, homologues of which are involved in the formation of PAPS (Table 2). APKS phosphorylates adenylylsulfate, the product of ATP-sulfurylase, converting it to PAPS. Other biosynthetic gene clusters that result in sulfated secondary metabolites also contain genes required for the production of PAPS (45).

Decarbamoylated analogues of STX could be produced via either of two hypothetical scenarios. Enzymes that act downstream of SxtI, the carbamoyltransferase, in the biosynthesis of PSP toxins are proposed to exhibit broad substrate specificity, processing both carbamoylated and decarbamoylated precursors of STX. Alternatively, hydrolytic cleavage of the carbamoyl moiety from STX or its precursors may occur. SxtL is related to GDSL lipases, which are multifunctional enzymes with thioesterase, arylesterase, protease, and lysophospholipase activities (1). The function of SxtL could therefore include the hydrolytic cleavage of the carbamoyl group from STX analogues.

Cluster-associated genes involved in metabolite transport. Kinetic studies of PSP toxin accumulation in producing cells and the media of cyanobacterial cultures suggest that there is an active transport mechanism for these toxins (9). In addition, variations in the concentration of sodium in culture media are known to affect the accumulation of PSP toxins in producer cells (41). *sxtF* and *sxtM* encoded two proteins with high sequence similarity to sodium-driven multidrug and toxic com-

pound extrusion (MATE) proteins of the NorM family. Members of the NorM family of MATE proteins are bacterial sodium-driven antiporters that export cationic substances (6). All of the PSP toxins are cationic substances, except for the C toxins, which are zwitterionic. It is therefore probable that SxtF and SxtM are also involved in the export of PSP toxins. A mutational study of NorM from *Vibrio parahaemolyticus* identified three conserved negatively charged residues (D32, E251, and D367) that confer substrate specificity; however, the mechanism of substrate recognition remains unknown (34). In SxtF, the residue corresponding to E251 of NorM is conserved, whereas those corresponding to D32 and D367 are replaced by the neutral amino acids asparagine and tyrosine, respectively. Residues corresponding to D32 and E251 are conserved in SxtM, but D367 is replaced by histidine. The changes in substrate-binding residues may reflect the differences in PSP toxin substrates transported by these proteins.

Putative transcriptional regulators of saxitoxin synthase. Environmental factors such as nitrogen and phosphate availability have been reported to regulate the production of PSP toxins in dinoflagellates and cyanobacteria (3, 10). Two transcriptional factors, *sxtY* and *sxtZ*, related to PhoU and OmpR, respectively, as well as a two-component regulator histidine kinase proximal to the 3′ end of the *sxt* gene cluster in *C. raciborskii* T3 have been identified. PhoU-related proteins are negative regulators of phosphate uptake (52), whereas OmpR-like proteins are involved in the regulation of a variety of metabolisms, including nitrogen (11) and osmotic balance (12). It is therefore likely that PSP toxin production in *C. raciborskii* T3 may be regulated at the transcriptional level in response to the availability of phosphate as well as other environmental factors.

Phylogenetic origins of the *sxt* genes. There is considerable interest regarding the phylogenetic origin of PSP toxin biosynthesis and how the capacity to produce these toxins has developed in such distantly related organisms, namely, dinoflagellates and bacteria (38). The *sxt* gene cluster from *C. raciborskii* T3 has a true mosaic structure. Approximately half of the *sxt* genes of *C. raciborskii* T3 were most similar to counterparts from other cyanobacteria; however, the remaining genes had their closest matches with homologues from proteobacteria, actinomycetes, sphingobacteria, and firmicutes. There is an increasing body of evidence indicating that horizontal gene transfer (HGT) is a major driving force behind the evolution of prokaryotic genomes (14), and cyanobacterial genomes are known to be greatly affected by HGT, often involving transposases and phages (35). The fact that the majority of *sxt* genes are most closely related to homologues from other cyanobacteria suggests that STX biosynthesis may have evolved in an ancestral cyanobacterium that successively acquired the remaining genes from other bacteria via HGT. The structural organization of the investigated *sxt* gene cluster, as well as the presence of several transposases related to the IS*4* family (8), suggests that small cassettes of *sxt* genes are mobile. The reasons for the restricted distribution of these biosynthetic genes to only four species in two orders of the *Cyanobacteria* are unknown but are critical for understanding the evolution of additional toxigenic species in the future. Sequencing of the *sxt* genes from several dinoflagellate species is under way and will assist in the further elucidation of the origin and evolution of these genes.

The constitutive expression of *sxt* genes in PSP toxin-producing cyanobacterial strains via reverse transcriptase PCR has been verified (data not shown), and the transcriptional regulation of the biosynthetic cluster is also under investigation. These experiments will aid the elucidation of the physiological function of the saxitoxins in bacteria and algae.

Saxitoxin synthase is a complex multienzyme pathway involving a novel PKS. The discovery of these genes will enable the rapid and accurate detection of harmful PSP-producing species in water and seafood. Intermediate compound analysis by MS and in vitro biosynthesis confirmed the sequence of reactions. Saxitoxin synthesis is the first nonterpene alkaloid pathway described for bacteria. Apart from understanding the functional role of these secondary metabolites and the factors that regulate their expression in the environment, accurate descriptions of the architecture of this complex system should provide strategies for combinatorial biosynthesis to generate novel neuroactive products.

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